CYTOLYTIC ACTION OF NORMAL AND IMMUNE SERUM ON INFUSORIA

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INTRODUCTION

The normal occurrence in the blood of various animals of bodies destructive to the cells of other animal species is generally recognized. It has been shown, for instance (H. Windsor), that a substance hemolytic for guinea-pig corpuscles occurs in human serum. We know also that fresh serum of many, if not all, animals contains a toxic substance, which causes contraction of smooth muscle; this toxic property diminishes considerably when the serum is kept and has nothing to do with the anaphylactic reaction.[1]

We have found no systematic observation concerning the action of "cytolytic" or toxic substance or substances in normal serums of various animals on different kinds of infusoria. Rössle,[2] however, made some observations on the production of antibodies to infusoria, and in the course of this work noted a few facts regarding the action of normal serum on paramecium. He compared the action of a 5-10 times dilution of normal and specific immune serums on paramecia, and found intensive paralyzing of paramecia to be the specific toxic action, but he did not find any definite phenomena which could be regarded as analogous to bacteriolysis. It must be especially noted here that Rössle used for the dilution of the normal and immune serums the liquid of his paramecia-culture, which was prepared without adding any particular electrolytes (salts).

The present work was designed principally to investigate the influence of normal animal serums on the monocellular organisms, such as protozoa, which are large and easily distinguishable whether they are active or dead.

METHOD AND TECHNIC EMPLOYED

Clear active or heated serums were diluted and distributed in series so as to determine the smallest amount capable of killing about the same number of infusoria in a definite time. Using small test tubes, a series of several dilutions of serum with 0.6% sodium chlorid solution was made. To each dilution of serum an equal amount of paramecia suspension in water was added. The time

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[1] Bayliss, W. H.: Principles of General Physiology, 1915, p. 730.

[2] Arch. f. Hyg., 1905, 54, p. 1.

of this addition was noted, and after 10, 15 and 60 minutes or 24 hours, the paramecia in tubes were microscopically examined to note their movement and any alteration in their form. Usually the tubes were incubated at 34-35 C., but in a few instances were kept at room temperature.

By means of a small capillary pipet, a small amount of liquid containing the protozoa was withdrawn from the lower layer, much care being taken not to destroy paramecia with the end of the pipet. One to two drops of this liquid were put on a slide and the activity of motion, morphologic alterations of paramecia, such as vacuoles, swelling of body, discharge of trichocysts and disintegration of the protoplasm, were microscopically observed with a Spencer objective 16 mm. and 4 mm. and ocular 10 X.

The culture of paramecia (Paramaecium caudatum) was obtained from Dr. Libble H. Hyman, Hull Zoological Laboratory of the University of Chicago, who kindly gave me many suggestions about the methods of infusoria culture. The culture was grown as follows:

Hay infusion, diluted with tap water, was sterilized with some hay. In a sterile jar containing about 400 c c of the diluted infusion, I transfered several cubic centimeters of the original paramecia culture, containing several kinds of bacteria on which the paramecia feed. This was kept at room temperature between 21 and 24 C., sometimes at incubator temperature (Rössle). The paramecia may appear abundantly in a week after starting the culture and can be used for the present purpose.

Three strains of paramecia (Paramaecium caudatum) were used as follows: (1) Paramecia alone, without any other kinds of infusoria in the culture; (2) a culture of paramecia with a small number of colpoda, and (3) a paramecia culture with very small number of stylonichyia.

Suspension of paramecia: Instead of using the culture liquid itself for the experiments I used paramecia suspension in water made as follows: a heavy paramecia culture is poured into a large, sterile, narrow-mouth flask, containing a sufficient amount of sterilized tap water, bringing the surface of the water very close to the neck of the flask. Within a few minutes almost all the paramecia come up to the surface and can be collected easily with a clean pipet. Repeating this process several times, using each time a fresh sterile flask with sterilized tap water at room temperature, any other substances in the original culture liquid which might affect in any way the action of the substances in the serum on paramecia can be almost entirely eliminated.

To count the number of paramecia contained in a suspension, 1 c c or more was mixed with 10 c c of about 20% pure gelatin and plated in a Petri dish in which the number of animals can be counted just as colonies of bacteria are counted.

The number of paramecia in a suspension can be counted quite easily in a narrow tube (a thin walled 1 c c pipet graduated in 100ths), holding the pipet horizontally and closing the upper end by a rubber bulb. The number of paramecia swimming in the liquid can be determined very easily, if there are not too many animals. If the protozoa are too numerous the suspension may be diluted with water. Throughout this work I used a suspension of from 100 to 120 paramecia in 1 c c of water.

In the following tests with older serums microscopic examination and cultivation with agar agar plate were made each time to avoid any error which might be caused by the use of spoiled or contaminated serum. Because of the strong injurious action of all kinds of disinfectants on paramecia (swelling, discharge of trichocysts and disintegration) none of them was used for the purpose of keeping the serum.[3]

[3] Kölsch: Zoolog. Jahrbücher, 1902, 16, p. 273.

SALTS AND THEIR TOXICITY AGAINST PARAMECIA

The great significance of the kinds of salts and their concentration in biologic experiments does not need any further explanation.[4, 5]

All serologic reactions are dependent to a great extent on the presence of electrolytes in the medium. Calcium sulphate and barium sulphate interfere with the action of complement (Hektoen and

TABLE 1*

RESULTS OBTAINED IN THE EXAMINATION OF THE INDIVIDUAL SALTS

		1	2	3	4	5	6	7
M/5 salt solution in distilled water		0.5	0.4	0.3	0.2	0.15	0.1	—
Distilled water		—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia		0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Time of Observation							
$MgSO_4.7H_2O$	15 minutes	+	++	+++	+++	+++	+++	+++
	30 minutes	†	†	++	+++	+++	+++	+++
	60 minutes	†	†	†	+++	+++	+++	+++
	20 hours	†	†	†	+++	+++	+++	+++
NH_4Cl	15 minutes	†	+†	++	+++	+++	+++	+++
	30 minutes	†	†	+†	+++	+++	+++	+++
	60 minutes	†	†	+†	++	+++	+++	+++
	20 hours	†	†	†	†	+†	++	+++
$BaCl_2$§	17 minutes	†	†	†	†	†	†	+++
KBr	15 minutes	†	+†	++	+++	+++	+++	+++
	30 minutes	†	+†	++	+++	+++	+++	+++
	60 minutes	†	†	++	+++	+++	+++	+++
	20 hours	†	†	†	+†	+++	+++	+++
NaBr	15 minutes	†	+†	+†	+++	+++	+++	+++
	30 minutes	†	†	+†	+++	+++	+++	+++
	60 minutes	†	†	†	++	+++	+++	+++
	20 hours	†	†	†	+	+++	+++	+++
NaI	15 minutes	†	+++	+++	+++	+++	+++	+++
	30 minutes	†	++	+++	+++	+++	+++	+++
	60 minutes	†	†	++	+++	+++	+++	+++
	20 hours	†	†	†	+++	+++	+++	+++
NaCl	15 minutes	†	+	+++	+++	+++	+++	+++
	30 minutes	†	+†	++	+++	+++	+++	+++
	24 hours	†	†	++	+++	+++	+++	+++

* +++ all animals motile. ++ some of them are motile. + very few of them are motile, the others are nonmotile. +† almost all of the animals are precipitated and very few of them are slowly motile. † all animals are precipitated and nonmotile.

§ This result indicates that Ba salt is exceedingly poisonous for the infusoria, as many investigators have stated.

Ruediger). Magnesium sulphate, calcium chlorid, potassium iodid, sodium iodid and barium chlorid are especially unfavorable for the complement action (Friedberger, v. Dungern and Coca, Pribram). According to Gengou, sodium citrate checks the complement action to

[4] Loeb, J.: Pflüger Arch., 1903, 97, p. 394.

[5] Loeb, J., and Wasteny, Hardolph: Biochem. Ztschr., 1911, 32, p. 155.

some extent. Recently Noguchi studied this phenomenon systematically.[6]

Paramecium itself does not require any particular salt to be added to the medium in which the animal is living; but these animals must be put into a dilution of serum and as we shall see later, the toxic action of normal serum on paramecia depends very much on the salt present. Here we have to choose salts and a concentration, which shall not be injurious to these animals and which shall be favorable to the action of serum on paramecia.

The following salts were examined individually with their M/5 solution with respect to their action on paramecia: $MgSO_4.7H_2O$; NH_4Cl; $BaCl_2$; NaBr.2HO; NaI and NaCl.[7]

Altho the microscopic examination was made in each case in the experiments previously described, only the result of M/5 NaCl will be given, since the others are more or less similar to this.

Microscopic Examination:

1. Tube 1: 7 minutes after the addition of suspension of paramecia. All animals are flat, have many vacuoles in their bodies. They are, however, all actively swimming around; no marked discharge of trichocysts. The anterior end of the body is injured most.

2. Tube 2: 17 minutes. Almost the same picture as above.

3. Tube 1: 30 minutes. Many animals have 2 large vacuoles and numerous smaller ones in their bodies. Their bodies markedly flattened. The cilia on the surface of the anterior half of the body are almost motionless. Trichocyst discharge is not marked.

4. Tube 2: 33 minutes. All animals are swollen. Some of them have discharged trichocysts around themselves.

5. Tube 3: 35 minutes. All animals are swimming around very actively, with number of small vacuoles in their body. No other remarkable change.

From this experiment with M/5 NaCl solution, we see that NaCl is less toxic than other salts, and 0.3 c c (Tube 3) of M/5 NaCl solution is only slightly injurious to paramecia. Repeated tests with various concentrations of this salt have shown that 0.6% solution is the rational concentration for our present purpose, that is, the solution of NaCl of that concentration, diluted again to two by the suspension of paramecia, is not injurious to paramecia at all, and, at the same time it does not depress the action of normal serum on paramecia. Therefore the 0.6% solution of NaCl was used for the dilution of serum every time. In each titration 0.5 c c of serum dilution with 0.6% NaCl solution and 0.5 c c of suspension of paramecia in water were used; the approximate salt concentration in every tube was about 0.3%.

[6] Biochem. Ztschr. 1907, 6, p. 172.

[7] Lösch, Karl: Zoolog. Jahrbücher, 16, 1902, p. 357.

A. ACTION OF NORMAL SERUMS OF WARM-BLOODED ANIMALS ON PARAMECIA

(*a*) Human Serum: Human blood was taken just before lunch in 6 tubes each containing 5-6 c c blood.

Two series of serum dilution were tested 3 hours later, one at room temperature, the other in the incubator for 1 hour and then at room temperature. As in many other cases the human serum acts on paramecia very much quicker at incubator temperature than at room temperature. At the end of 1 hour the first examination was made, and after that time both series were put at room temperature for about 20-24 hours, when the second examination was made.

TABLE 2

RESULTS OBTAINED IN THE TESTS WITH HUMAN SERUM

	1	2	3	4	5	6
Active human normal serum, diluted with 0.6 % NaCl solution to 1 : 5....	0.4	0.3	0.2	0.15	0.1	—
0.6 % NaCl solution.....	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia.....	0.5	0.5	0.5	0.5	0.5	0.5
Series 1.—At room temperature: Time of observation—						
30 minutes.....	+†	++	+++	+++	+++	+++
45 minutes.....	†	+†	+†	+++	+++	+++
20 hours.....	†	†	†	+†	+++	+++
Series 2.—At the incubator for 1 hour, then at room temperature: Time of observation—						
30 minutes.....	+†	+†	+†	+++	+++	+++
45 minutes.....	†	†	+†	+†	+++	+++
20 hours.....	†	†	†	+†	+++	+++

Microscopic examination at the end of one hour.

Series 1: Observation at room temperature.

Tube 1: Very little disintegration and discharge of trichocyst. Some are still motile, but swollen.

Tube 2: Some animals are swollen, and slowly motile.

Tube 3: All animals are actively motile.

Series 2. Observed after 1 hour in the incubator.

Tube 1: All animals are disintegrated; trichocysts discharged. Colpoda are still very actively swimming around.

Tube 2: The picture is similar to Tube 1.

Tube 3: Some animals are in active locomotion, but move very slowly. Few of them are already disintegrated. Others are swollen, with trichocysts discharged. Colpoda still alive in active condition.

Microscopic examination of both series after 24 hours at room temperature shows same result.

1. Tube 1: Very few visible residues of disintegrated paramecia. Even single colpoda not to be seen.

2. Tube 2: Same as Tube 1.

3. Tube 3: Very few paramecia in disintegrated condition. Colpoda are very actively swimming around in the medium.

4. Tube 4: Almost all paramecia are disintegrated. Very active locomotion of colpoda.

5. Tube 5: Paramecia actively motile, with many vacuoles. Some of them are already disintegrated. Many colpoda are very actively swimming around.

6. Tube 6: All paramecia and colpoda are in active condition.

Colpoda which were simultaneously cultivated with paramecia and came into the suspension, are more resistant against human blood serum than paramecia.

The titer of fresh human blood serum lies between Tubes 3 and 4; that is, between 0.2 and 0.15 c c of 1 : 5 dilution.

The blood serum specimen kept at room temperature lost its activity more rapidly than that kept in the ice-box.

The titration of another serum specimen taken on Jan. 12, 1918, 2 hours after luncheon, showed that there was no definite relation between the amount of the toxic substance in the blood at different periods.

The human serum heated for one-half hour at 56 C. was tested in the same manner as before. After 24 hours all animals in the medium in every tube were swimming around actively; there was no sign of toxic action of inactivated serum on infusoria.

TABLE 3

RESULTS OBSERVED IN THE TESTS WITH SERUMS FROM DIFFERENT ANIMALS

Serum	Titer against the Same Number of Paramecia	
	Dilution	Amount, c c
Human	1 : 5	0.2 : 0.15
Horse	1 : 5	0.2 : 0.15
Sheep	1 : 5	0.15 : 0.1
Beef	1 : 20	0.2 : 0.15
Hog	1 : 20	0.4 : 0.3
Guinea-pig*	1 : 20	0.3 : 0.2
Rabbit	1 : 20	0.3 : 0.2
Pigeon	1 : 10	0.2 : 0.1

* I noted some individual variation.

As in the case of carbolic acid test, paramecia, acted on by human serum, discharge trichocysts[8] in the medium. These can be seen distinctly under the low power of the microscope.

Several normal human serums were obtained and titrated with almost the same result. Each specimen was carefully tested for Wassermann reaction with negative result.

There is no difference in resistance against normal serum between paramecia raised at room temperature and those cultivated at incubator temperature, if, in making the suspension, care is taken to avoid sudden changes of temperature of the medium.

Human normal spinal fluid, which is said to have no complement (MacKenzie and Martin, Houston, Hektoen) was tested in the same way with the infusoria, but no sign of toxic action was observed.

[8] The discharge of trichocysts under the influence of stimuli has been studied by Massart (1901) and by Statkewitsch (1903). Crushing the animal causes discharge of trichocysts in the region of injury. Weaker mechanical stimuli do not have this effect. If the animal is heated rapidly till it is killed, it discharges the trichocysts before dying; if heated slowly, this effect is not produced. Neither cold nor increased pressure have any effect on trichocysts. Many chemicals, particularly acids, produce the discharge. An electrical induction shock causes the same effect; when it is weak, the discharge is at the anode only; stronger shock causes discharge at both anode and cathode; still stronger shock causes discharge of trichocysts over the entire surface of the body (Statkewitsch 1903). (Jennings: Behavior of Lower Organisms, p. 89.)

(*b*) Serums of Other Warm-Blooded Animals: Using the same method normal serums of different warm-blooded animals were tested with the results given in Table 3.

In all tested serums of warm-blooded animals the activity against paramecia is entirely destroyed when they are heated at 56 C. for 20 minutes. For reactivation refer to Section 6. The microscopic alteration in the body of the protozoa is just the same as that observed in the case of human serum.

B. ACTION OF SERUM OF COLD-BLOODED ANIMALS

(*a*) Frog Blood Serum: Blood was taken direct from the heart. After centrifugalization clear serum separated, which was titrated as usual.

TABLE 4

TESTS MADE WITH FROG BLOOD SERUM

	1	2	3	4	5	6	7
Active normal frog serum diluted with 0.6 % NaCl to 1 : 10	0.5	0.4	0.3	0.2	0.15	0.1	—
0.6 % NaCl solution	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation at room temperature:							
10 minutes	†	+†	+	++	++	++	+++
30 minutes	†	†	†	†	†	+†	+++

Microscopic examination after 1 hour and 25 minutes.

Tube 3: Disintegration; spherical shape; very marked trichocyst discharge. Two very large vacuoles in the body of other animals which are about to disintegrate.

Tube 4: Nonmotile; few are already disintegrated. Most of them are swollen and have a spherical shape, with two very large vacuoles in the body. Trichocyst discharge very marked.

TABLE 5

SHOWING THE SLIGHT INDIVIDUAL VARIATION IN THE ACTIVITY OF NORMAL FROG SERUM ON PARAMECIA

	1	2	3	4	5	6	7
Frog 5: Dilution of serum 1 : 50	0.5	0.4	0.3	0.2	0.15	0.1	—
Time of observation at room temperature—							
30 minutes	†	†	†	+†	++	+++	+++
60 minutes	†	†	†	†	++	+++	+++
18 hours	†	†	†	†	+	+++	+++
Frog 6: Time of observation at room temperature—							
30 minutes	†	†	+†	++	+++	+++	+++
60 minutes	†	†	+†	++	+++	+++	+++
18 hours	†	†	†	+†	+	+++	+++

Tube 5. All animals are entirely nonmotile. More or less swollen, but no disintegration to be seen.

Tube 6. Very slight motion of cilia in the cystostom still present.

A slight individual variation in the strength of action on paramecia of normal frog serum is recorded in Table 5. Blood was taken and tested on March 20, 1918.

The activity of normal frog serum on paramecia is entirely destroyed when the serum is heated at 56 C. for 20 minutes.

The blood serum of normal frog contains hemolysin against the corpuscles of rabbit.

Given corpuscles or protozoa succumb to the dissolving effects of the complement and lytic substance in the frog serum at different rates which cannot be directly compared with each other from the result obtained in each experiment, because the relation of the number of cells to the dissolving agencies is not the same in the two cases. In other words, we use about 0.5 c c of paramecia suspension, 1 c c of which contains about 100-120 protozoa, while in the hemolysis, we use 0.5 c c of 5% suspension of corpuscles in salt solution.

(*b*) Turtle Blood Serum (Chrysemys Picta): The blood was taken from arteria carotis of the turtle. Clear serum was titrated in the usual way, 0.6% NaCl solution being used for the dilution. The titer limit lies between 0.15 and 0.1 c c of 1:50 dilution. The clear serum was heated for 30 minutes to a temperature varying from 45-60 C., after which the activity of the serum was again noted.

TABLE 6

RESULTS OF TESTS MADE WITH TURTLE BLOOD SERUM

		1	2	3	4	5	6	7	8
Heated serum	Dilution 1 : 5....	0.5	0.4						
	Dilution 1 : 10...			0.5	0.4	0.3	0.2	0.1	—
	0.6 % NaCl.....	—	0.1	—	0.1	0.2	0.3	0.4	0.5
	Suspension of paramecia....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	Time of Observation at 23 C.								
Heated at 45 C. for 30 minutes	7 minutes.....	†	†	+†	+†	+++	+++	+++	+++
	15 minutes.....	†	†	†	†	+	+†	+	+++
	30 minutes.....	†	†	†	†	†	†	†	+++
Heated at 50 C. for 30 minutes									
	30 minutes.....	+++	+++	+++	+++	+++	+++	+++	+++
	60 minutes.....	+++	+++	+++	+++	+++	+++	+++	+++
	2 hours.....	+++	+++	+++	+++	+++	+++	+++	+++
	18 hours.....	†	+†	+†	++	+++	+++	+++	+++
Heated at 53 C. for 20 minutes									
	30 minutes.....	+++	+++	+++	+++				
	4 hours.....	+++	+++	+++	+++				
	24 hours.....	+++	+++	+++	+++				

According to these results heating at 50 C. for 30 minutes is not sufficient to cause a complete inactivation of the complement against paramecia in the normal turtle serum; for this it is necessary to heat the serum at 53 C. for 20 minutes.

Repeated titration with dilution of 1:50 shows that by heating the turtle serum at 45 C. for 30 minutes, the complement in the serum is affected only a little; the titer on paramecia is almost the same as the original serum*

*The turtle serum contains an appreciable amount of natural hemolysin against rabbit corpuscles. A more detailed description will be given in a subsequent paper.

ADSORPTION TEST

As stated by v. Dungern, Landsteiner and Eisner,[9] Andrejew[10] and other investigators, substances in blood serums can be adsorbed by many kinds of adsorbents, such as casein, quartz sand, coal, barium sulsulphate, kaolin, etc.

1. *Adsorption by Bone Black.*—Five c c normal serum was mixed with 0.5 gm. charcoal and after shaking thoroughly in a tube, was put in the incubator for 1 hour at 36 C. The serum was then centrifugalized for about 10 minutes and the clear serum used in titration. As a control experiment, the same amount of serum was kept in the same incubator for the same time and centrifugalized and titrated in the same manner.

(*a*) Adsorption of horse serum by bone black. The serum was kept for 48 hours in the ice-box. For the dilution of the serum 0.6% NaCl solution was used.

TABLE 7

RESULTS OF TESTS MADE WITH BONE BLACK

	1	2	3	4	5	6	7
Adsorbed horse serum diluted to 1 : 5 with 0.6 % NaCl solution.....	0.5	0.4	0.3	0.2	0.15	0.1	—
0.6 % NaCl solution.....	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia....	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
20 minutes.....	†	++	+++	+++	+++	+++	+++
60 minutes.....	†	++	++	+++	+++	+++	+++
20 hours.....	†	++	+	++	++	+++	+++
Control serum:							
20 minutes.....	†	++	++	+++	+++	+++	+++
60 minutes.....	†	†	†	+++	+++	+++	+++
20 hours.....	†	†	†	++	+++	+++	+++

(*b*) Adsorption of beef serum by bone black. The serum was kept for 48 hours in the ice-box.

The titer of the adsorbed beef serum is between 0.3 and 0.2 c c of 1:20 dilution, while that of the control experiment is between 0.15 and 0.1 c c of 1:20 dilution of normal beef serum.

2. *Adsorption by Infusorial Earth.*—Five c c of 10% suspension of infusorial earth in distilled water and 5 c c of normal beef serum, 120 hours old, were shaken thoroughly and the mixture put in the incubator for an hour and then centrifugalized for about 10 minutes. This clear serum, being diluted already to 1:1 with distilled water, ought to be diluted further to 1:20 with salt solution and the final salt concentration should be 0.6 % NaCl.

The adsorbents used were labeled chemically pure, and repeated experiments showed no substances present that were injurious to the infusoria.

[9] Wiener klin. Wchnschr., 1904, 24, p. 676.

[10] Arbeit a. d. k. Gesndthsamte, 1909, 33, p. 84.

TABLE 8

RESULTS OF TESTS MADE WITH INFUSORIAL EARTH

	1	2	3	4	5	6	7
Adsorbed normal beef serum 1 : 20 dilution with 0.6 % NaCl	0.5	0.4	0.3	0.2	0.15	0.1	—
After 20 hours	†	†*	+++	+++#	+++	+++	+++

* Denotes the position of the titer limit of the adsorbed serum, while # denotes that of control test with normal beef serum without any adsorption. A marked difference between these two titer limits is noted.

Owing to the low titer of normal serum it was not possible to determine any mathematical relation concerning the law of adsorption in modern interpretation.

These results show that the toxic substance or substances which act on paramecia can be adsorbed by some adsorbents.

REACTIVATION

In each case of titration of normal serum, the latter loses the greater part of its toxic properties by heating at 56 or 53 C. for half an hour.

In adding normal guinea-pig serum for reactivation, we must keep in mind the fact that the normal serum of guinea-pig itself exerts a strong toxic action on paramecia.

1. *Reactivation of Inactivated Fresh Normal Beef Serum by Fresh Normal Guinea-Pig Serum.*—A very small amount of normal active beef serum, for instance, 0.15-0.10 c c of 1 : 20 dilution, is still capable of causing some paramecia to disintegrate. Assuming that the fresh beef serum has as much complement as the fresh guinea-pig serum, the reactivation test of inactivated beef serum was made.

TABLE 9

TESTS MADE WITH INACTIVATED BEEF SERUM

	1	2	3	4	5	6	7
Inactive beef serum diluted with 0.6 % NaCl to 1 : 20	0.4	0.3	0.2	0.15	0.1	0.5	—
0.6 % NaCl solution	—	0.2	0.2	0.25	0.3	—	0.3
Guinea-pig serum diluted to 1 : 20	0.1	0.1	0.1	0.1	0.1	—	0.2
Suspension of paramecia in water	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
1 hour	†	†	†	†	†	+++	+++
24 hours	†	†	†	†	†	+++	+++

Microscopic examination at the end of 1 hour gave a uniform result in each tube except the control test (Nos. 6 and 7); that is, all animals are entirely disintegrated. The result of this experiment is very remarkable and can be easily understood as in the case of bacteriolysis and hemolysis.

2. *Reactivation of Inactive Hog Serum by Fresh Normal Guinea-pig Serum.*—Fresh active hog serum in 0.1 c c of its 1 : 20 dilution is capable of diminishing the free mobility of paramecia and the inactive hog serum has no toxic action

on paramecia. I tried to test the possibility of reactivation of inactivated hog serum by the aid of active normal guinea-pig serum, with a distinctly smaller amount of the latter than the titer limit against paramecia, which is estimated by direct titration.

TABLE 10

TESTS MADE WITH INACTIVE HOG SERUM

	1	2	3	4	5	6	7
Inactive hog serum diluted with 0.6 % NaCl solution to 1 : 20	0.4	0.3	0.2	0.15	0.1	0.5	—
0.6 % NaCl solution	—	0.1	0.2	0.25	0.3	—	0.3
Active normal guinea-pig serum diluted with 0.6 % NaCl to 1 : 20	0.1	0.1	0.1	0.1	0.1	—	0.2
Suspension of paramecia in water	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
60 minutes	+†	+†	+†	+†	+†	+++	+++
2 hours	†	†	†	†	†	+++	+++
24 hours	†	†	†	†	†	+++	+++

This fresh hog serum alone in 0.1 c c of 1:20 dilution is not sufficient to destroy all paramecia: and this is due, at least to some extent, to a relatively insufficient amount of complement in it, while the inability of 0.1 c c of 1:20 dilution of fresh guinea-pig serum to disintegrate the same number of paramecia, is due to a relatively insufficient amount of lytic substance (amboceptor, which is thermostabile) in the normal guinea-pig serum.

The last statement, that in the guinea-pig serum the lytic substance (normal amboceptor) is insufficient in amount to combine with the whole amount of complement, is proved by the experiment described in Table 11.

TABLE 11

TESTS PROVING THAT IN GUINEA-PIG SERUM THE LYTIC SUBSTANCE IS INSUFFICIENT IN AMOUNT TO PROPERLY COMBINE

	1	2	3	4	5	6	7	8	9
Guinea-pig serum diluted to 1 : 20 with 0.6 % NaCl	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.2	0.3
0.6 % NaCl solution	—	0.05	0.1	0.15	0.2	0.25	0.35	0.3	0.2
Inactive beef serum diluted with 0.6 % NaCl solution to 1 : 50	0.35	0.3	0.25	0.2	0.15	0.1	—	—	—
Suspension of paramecia	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hour in the incubator and then at room temperature):									
60 minutes	+†	+†	++	++	+++	+++	+++	+++	+++
16 hours	†	†	†	†	†	+	++	+++	+++
17 hours	D	D	D	D	D—				

Microscopic examination at the end of 17 hours. D denotes entire disintegration of paramecia; D— denotes incomplete disintegration of paramecia.

While 0.15 c c of 1 : 20 dilution of fresh normal guinea-pig serum is unable by itself to kill the paramecia, the same amount of fresh normal guinea-pig serum mixed with a sufficient amount (over 0.15 c c of 1 : 50 dilution) of inactive beef serum, containing normal amboceptor only, is able to kill the paramecia entirely; in other words, the insufficient amount of normal amboceptor needed to fix the corresponding amount of complement present in the fresh guinea-pig serum is supplied in this case by the inactivated beef serum.

5. *Reactivation of Inactive Rabbit Serum by Fresh Normal Guinea-Pig Serum.*—I observed a phenomenon which seems to be analogous to the "complement deviation," in the estimation of normal amboceptor in normal rabbit serum, as seen in Table 12:

TABLE 12

DETAILS REGARDING A PHENOMENON OBSERVED DURING THE TESTS

	1	2	3	4	5	6	7	8	9	10	11
Inactive rabbit serum diluted with 0.6 % NaCl to 1 : 5.....	0.4	0.3	0.2	0.15							
To 1 : 10.....					0.3	0.2	0.15				
To 1 : 20.....								0.3	0.2	0.15	—
0.6 % NaCl.....	—	0.1	0.2	0.25	0.1	0.2	0.25	0.1	0.2	0.25	0.5
Fresh normal guinea-pig serum 1 : 20.....	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	—
Suspension of paramecia in water.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation:											
1 hour.....	+++	+++	+++	+++	+++	†	†	†	+	++	+++
20 hours.....	+++	+++	++	++	†	†	†	†	†	+	+++

The titer limit of normal guinea-pig serum used in this test as the source of complement is between 0.2 and 0.15 c c of 1:20 dilution; 0.1 c c of that dilution is certainly incapable of killing the number of paramecia present within 20 hours.

This examination was repeated several times with different serums and the result was always the same. In all tubes in which the amount of amboceptor is in excess, there is no action of the serum on paramecia, a phenomenon analogous to the deviation of complement according to Neisser and Wechsberg.

The same "prozone" is observed also when immune rabbit serums are used instead of normal serum; but, contrary to our expectation, the extent of the "prozone" is not parallel with the amount of immune amboceptor in the immune serum; that is, the difference in the extent of the "prozone" of normal and immune serum is rather faint. We expect to discuss this matter more fully from the point of view of the theory of complement deviation. In our case "agglutination" can not be considered as a cause of this phenomenon as it is thought to be by some authors in the case of bacteriolysis,[11] because we do not see any sign of agglutination of paramecia in any one of the tubes in the "prozone" of this experiment.

4. *Reactivation Test of the Inactive Turtle Serum by the Normal Guinea-Pig Serum.*—Normal turtle serum was inactivated by heating at 53 C. for 20 minutes in a water bath. Normal guinea-pig serum was used as the source of complement.

[11] Buxton: Jour. Med. Research, 1905, 13, p. 431.

TABLE 13*

TESTS MADE WITH INACTIVE TURTLE SERUM

	1	2	3	4	5	6	7	8	9	10
Inactive turtle serum, diluted with 0.6 % NaCl to 1 : 5	0.4	0.3								
To 1 : 10			0.4	0.3						
To 1 : 20					0.4	0.3				
To 1 : 50							0.4	0.3		
To 1 : 100									0.4	0.3
0.6 % NaCl	—	0.1	—	0.1	—	0.1	—	0.1	—	0.1
Guinea-pig serum diluted with 0.6 % NaCl to 1 : 30§	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Suspension of paramecia	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
After 24 hours	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

* The control test tubes are omitted.

§ The smallest amount of normal guinea-pig serum capable of killing a given number of paramecia in a definite length of time was previously determined in the usual way. The amount used in this case is about one-half of the titer limit.

After 2-4 hours all the animals in all the tubes were swimming around vigorously; no marked sign of toxic action. Probably the normal amboceptor in the turtle serum is unable to be complemented by the normal complement in the guinea-pig serum. The normal hemolytic amboceptor against rabbit corpuscles in the turtle serum is not complemented at all by the normal guinea-pig serum.[12]

5. *The Lytic Amboceptor Against Paramecia in the Inactivated Normal Frog Serum.*—The lytic amboceptor against paramecia tested under these conditions was also unable to be reactivated by the addition of normal guinea-pig serum.

6. *Reactivation of Inactive Turtle Serum with Normal Frog Serum.*—The reactivation of inactive turtle serum by the normal active frog serum was attempted, but positive complementation was not observed. The hemolytic amboceptor in the normal turtle serum, which will be fully discussed in the subsequent paper, is also unable to be complemented by the normal complement of frog serum.[13]

SALT CONCENTRATION AND THE ACTION OF SERUM

As in other serologic reactions salt concentration of the medium is of great importance in the toxic action of normal serum on paramecia. Too high salt concentration and too low electrolyte concentration are both unfavorable for the toxic action of normal serum on paramecia, as indicated by the results of the following experiment.

Three series of tubes containing different dilutions of serum with different amounts of salt are compared in their titer of toxic action on paramecia.

In this case there is no sign of toxic action of beef serum; all animals in all tubes are active even after 47 hours at room temperature.

[12] Ritz: Ztschr. f. Immunitätsforsch., 1911, 9, p. 321.

[13] Noguchi, H.: Bull. Univ. of Pa., 1902, 15, p. 301.

TABLE 14

SALT CONCENTRATION TESTS. SERIES 1

	1	2	3	4	5	6	7
Beef serum diluted with distilled water to 1 : 20.....	0.5	0.4	0.3	0.2	0.15	0.1	—
1 c c of 0.85 % NaCl diluted with distilled water to 1 : 20.....	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia in water.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5
After 2 - 4 hours.....	+++	+++	+++	+++	+++	+++	+++

TABLE 15

SALT CONCENTRATION TESTS. SERIES 2

	1	2	3	4	5	6	7
Beef serum diluted with 0.3 % NaCl solution to 1 : 20..	0.5	0.4	0.3	0.2	0.15	0.1	—
1 c c of 0.85 % NaCl diluted with 0.3 % NaCl solution to 1 : 20.....	—	0.1	0.2	0.3	0.35	0.4	0.5
Suspension of paramecia in water.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):							
20 minutes.....	+†	+†	++	+++	+++	+++	+++
40 minutes.....	†	†	†	†	+++	+++	+++
60 minutes.....	†	†	†	†	+++	+++	+++
20 hours.....	†	†	†	†	+++	+++	+++

TABLE 16

SALT CONCENTRATION TESTS. SERIES 3

0.6 % NaCl solution is used instead of 0.3 % in the test in Series 2.							
20 minutes.....	†	†	+†	+++	+++	+++	+++
40 minutes.....	†	†	†	+†	+++	+++	+++
60 minutes.....	†	†	†	†	+++	+++	+++
20 hours.....	†	†	†	†	+†	+++	+++

The microscopic examination of the second and third series 1 hour after the addition of the suspension of paramecia shows almost the same degree of morphologic alteration of paramecia.

No sign of toxic action is apparent in the first series, where the salt concentration is low. In the second series the result of the serum action is almost the same as in the third series, where the salt concentration is as high as in many other series of experiments with normal serum on paramecia. A slight difference in the rapidity of action between the two series is noted, the action in the second series being somewhat slower than that in the third. Our result agrees exactly with that obtained by Buchner in his work on the bactericidal substance of normal serum. According to his findings, this can be destroyed by dilution of serum over 12 times with water and by dialysis against water (not against salt solution), and it can be reduced again by addition of salt solution.

Using the paramecia-immune rabbit serum, we obtained the same results, which indicates the necessity of electrolyte for the action of amboceptor and complement on paramecia.

The negative result obtained by Rössle (no lytic action of normal serum on paramecia) is probably due to the fact that he neglected the suitable concentration of electrolytes in the serum dilution.

IMMUNIZATION AGAINST PARAMECIA

Material for Immunization: A 10 days' culture of paramecia, containing large numbers, was subjected to an electric current to collect them and, at the same time, to get rid of as many of the contained bacteria as possible. A glass tube, 0.5 inch in diameter and of U form, both perpendicular parts of it having about the same length as the transverse part (= about 5 inches), was held firmly by a buret stand, and filled with paramecia culture, and a weak current was passed from one end of the tube to the other. I used 6 dry cells, each of which is of 1.5 volt, arranged by wire with an ammeter; the positive and negative poles were suspended in the liquid culture at the end of the tube. Under the above mentioned potential difference, I noticed 1/10 milliampere of current passing through the circuit. As soon as the circuit was closed the paramecia in the tube went over en masse to the cathode pole, with measurable speed. At first some paramecia remained around the anode pole or near the liquid surface, but after a short time all paramecia went over to the cathode. Some of them discharged trichocysts at the anode, that is, at the posterior end, and later were deprived of their motility and precipitate.

TABLE 17

RESULTS OF TESTS TO DETERMINE THE ACTION OF IMMUNIZED RABBIT SERUM ON PARAMECIA

	1	2	3	4	5	6	7
Rabbit serum 1, diluted with 0.6 % NaCl solution to 1 : 20	0.5	0.4	0.3	0.2	0.15	0.1	—
Action on paramecia in (1 hour in the incubator and then at room temperature):							
18 minutes	+†	+†	+†	+++	+++	+++	+++
25 minutes	†	†	†	+†	+++	+++	+++
45 minutes	†	†	†	+†	+†	+†	+++
60 minutes	†	†	†	+†	+†	+†	+++
20 hours	†	†	†	†	†	+†	+++
Microscopic examination at the end of 1 hour	D	D	D	D—			

If the cathode pole is held somewhat deep in the liquid, so that the paramecia pass through the cathode pole and gather near the surface of the liquid of that end in a large aggregation, most of the paramecia can be saved from the danger of injury by the current and many of them can be collected in a smaller amount of liquid. Repeating this process several times the bacteria in paramecia culture are eliminated almost completely.

By means of a centrifuge almost all the bacteria can be eliminated.

To each 0.1 c c of paramecia mass taken from the bottom of the centrifuge tube was added 1 c c of 0.6% NaCl solution and the thick suspension of paramecia was used as antigen. Three rabbits were used for immunization against paramecia. The antigen was given intraperitoneally in all 3 rabbits, weighing about 2,200 gm. each, 2.5 c c on Jan. 29, 3 c c Feb. 4, and again Feb. 14.

Through the whole course of the immunization we did not observe any particular symptom which could be regarded as an accidental result of the paramecia injection.

Action of Immunized Rabbit Serum on Paramecia: Blood was taken from the vein 5 days after the second injection of paramecia suspension. Clear serum was titrated immediately after the centrifugalization.

Microscopic examination at the end of 1 hour: Tubes 1-3 show an entire disintegration of all animals (indicated by D in Table 18), while in Tube 4 there are some animals swollen, with very large vacuoles; others make very slow movements (D—).

The smallest amount of the serum capable of killing the paramecia is 0.15-0.1 c c of 1:20 dilution. In all three of the immune serums tested results almost alike were obtained.

The same immune serum lost its action entirely by heating at 56 C. for 20-25 minutes, just as in the case of normal serum. Standing in an ice-box for 5 days, the active immune rabbit serum diminishes its titer to some extent, namely, the titer limit becomes 0.20 c c-0.15 c c of 1:20 dilution, and is caused by a diminished amount of complement.

On Feb. 20 other blood specimens were taken and titrated just as before; the titer limit of all 3 immune rabbit serums was between 0.2 c c and 0.15 c c of its 1:40 dilution.

TABLE 18

RESULTS OF TESTS IN ESTIMATING THE AMBOCEPTOR IN IMMUNE SERUM

	1	2	3	4	5	6	7	8	9	10	11
Inactive immune rabbit serum 1, diluted to 1 : 50....	0.4	0.3	0.2	0.15							
To 1 : 100.....					0.3	0.2	0.15				
To 1 : 200.....								0.3	0.2	0.15	—
0.6 % NaCl solution.....	—	0.1	0.2	0.25	0.1	0.2	0.25	0.1	0.2	0.25	0.5
Normal guinea-pig serum, diluted with 0.6 % NaCl to 1 : 20.....	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	—
Suspension of paramecia in water.....	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Time of observation (1 hr. in the incubator and then at room temperature):											
1 hour.....	†	†	†	†	†	†	†	+†	+†	+	+++
20 hours.....	†	†	†	†	†	†	†	†	†	+†	+++

Estimation of the Amboceptor in the Immune Serum: Blood specimens were taken from immunized rabbits 15 days after the third injection of paramecia. Clear serum was inactivated.

The titer limit of normal guinea-pig serum, which is to be used as complement source, is in this case between 0.2 and 0.15 c c of its 1:20 dilution; 0.10 c c of that dilution is certainly incapable of killing the number of paramecia within 24 hours.

According to the results at least 0.2 c c of 1:200 dilution of inactivated immune serum, containing amboceptor only, to cover the shortage on amboceptor (normal amboceptor) in guinea-pig serum for combining with complement present in 0.10 c c of 1:20 dilution of fresh normal guinea-pig serum, should be added, to enable it to kill the number of paramecia used.

By parallel estimation of normal amboceptor in normal rabbit serum using the method previously described, we find that we have to add at least 0.2 c c of 1:20 dilution of inactive normal rabbit serum to cover the deficiency of amboceptor in 0.1 c c of 1:20 dilution of guinea-pig serum.

The different data regarding the amount of inactivated serum (that is, 0.2 c c of 1:200 dilution of the immune serums and 0.2 c c of 1:20 dilution of the normal rabbit serums) necessary to enable the same amount of normal guinea-pig serum (0.1 c c of 1:20 dilution) to act completely (cytolysis) give us the relation with regard to the amount of amboceptor in the immune and in the normal serum.

All three immune serums were tested by this method and each titer (used here only in a limited sense of the word) of amboceptor is respectively: Rabbit 1, 0.2 c c of 1:200 dilution; Rabbit 2, 0.2 c c of 1:200 dilution, and Rabbit 3, 0.3 c c of 1:400 dilution.

The immune serums contain about 10 or 15 times as much amboceptor as normal serum.

From the statements under the section on reactivation, it is evident that the action of normal (and immune) serum is a more complicated one than the action of ordinary salt alone.

The death of paramecia under the influence of serum may not be caused by amboceptor and complement. Certainly the disintegration of paramecia can be effected, just as in the case of hemolysis, by many other chemical, physical and toxic agencies.[14] * Excess of hydrogen or hydroxyl ion is injurious for paramecia. Normal blood is alkaline in reaction to litmus, the alkalinity being due to some carbonate. When examined according to physico-chemical methods the blood is found to be neutral, that is, it does not contain an excess of hydroxyl ions. The death of paramecia by the action of serums cannot be interpreted as due to the alkalinity (neutrality) of the serum.

The morphologic and physiologic alterations of paramecia under the influence of normal and immune serum depression of the motility, discharge of trichocysts, swelling of the body and finally disintegration — are surely due to the serum. I observed experimental facts which are to be interpreted as purely serologic, not merely toxicologic, namely, that serum, when heated at 56 C. for about half an hour, loses its activity, and that this can be regained (reactivated) by addition of fresh normal guinea-pig serum (as complement source); I noticed some special rôle of electrolyte in the action of serum on paramecia. Furthermore, I observed that an excess of inactivated serum (ambo-

[14] Rarratt, W.: Ztschr. f. allg. Physiol., 1904, 8, p. 438. Ibid., 1905, 5, p. 73. Bokorny, T.: Arch. f. Zellforschung, 1911, 7, p. 1. Neuhaus, H.: Arch. internat. d. pharmacol., 1910, 20, p. 393.

* As to the action of some bacterial toxins on paramecia I tried diphtheria and tetanus toxin and some culture filtrates of some other bacteria. Strong diphtheria and tetanus toxins have no effect on paramecia, even in 1:2 dilution. Culture filtrates of hemolytic staphylococcus and streptococcus (both 10 days cultures) also have no effect on paramecia. Cultures of B. pyocyaneus cause death of paramecia, but this seems to be due to the alkalinity of the filtrate, and not to any specific "pyocyanolysin"—an assumption which will be very easily proved by neutralization of the filtrate with N/20 HCl; the effect of the filtrate on paramecia is lost entirely when the filtrate is just completely neutralized. (See also E. O. Jordan, Jour. Med. Research, 1903, 10, p. 51.)

ceptor) mixed with an adequate amount of fresh normal guinea-pig serum (complement source) is rather unfavorable for the action of serum on paramecia — a phenomenon which is easily regarded as analogous to the so-called "deviation of complement" according to Neisser and Wechsberg.

The serum of the same species is, as a rule, most favorable for the complementation of the amboceptor (Ehrlich). H. Noguchi[15] reports that the immune hemolytic amboceptor (painted turtle immunized with the blood of Rana catesbiana) is entirely complemented by its own serum, slightly by the normal complement of speckled turtle, and not at all by the normal complement of bull-frog. The reactivation test of the inactive turtle and frog serums by normal guinea-pig complement, and of the former by the normal frog complement, illustrate this fact. We need not be discouraged, therefore, by the negative complementation in this case. Obviously, the complements in the normal turtle and frog serums do play an important part in causing death of infusoria by the action of these normal serums.

Tho I did not succeed in producing a highly potent antiserum against paramecia, the results support my opinion that the action of serum on paramecia is an entirely serologic, a beautiful example of cytolytic action of serum — a fundamentally different conception from that stated by Rössle. He says that phenomena which can be considered as analogous to bacteriolysis were not found at all, even when a low dilution of undiluted serum was used.

The failure of Rössle to recognize these phenomena which ultimately ought to be regarded as analogous to cytolysis may probably be due to the fact that he did not pay special attention to the significance of the electrolyte in the action of serum on paramecia.

RÉSUMÉ

Clear serum was diluted and distributed in a series of tubes to determine the smallest amount necessary to kill the same number of infusoria (Paramaecium caudatum).

Special attention was paid to the kind of salt and its concentration for the dilution of serum. Throughout the experiment 0.6% NaCl solution was used, unless special note is made.

The following kinds of serums were tested: human, horse, sheep, hog, beef, guinea-pig, rabbit, pigeon and frog and turtle.

[15] Bull. Univ. of Penn., 1902, 15, p. 301.

The toxic action of serum consists in depression of motility, discharge of trichocysts, swelling and finally disintegration of the bodies of paramecia. Colpoda is somewhat more resistant than Paramaecium.

Acting substance or substances can be adsorbed by various adsorbents.

The toxic activity disappears when serum is heated at 56 C. for about half an hour (warm-blooded animals) or at 53 C. for 20 minutes (cold-blooded animals). The reactivation was tested with fresh normal guinea-pig serum as complement source. Fresh guinea-pig serum contains relatively more complement than is required to combine with its own amboceptor.

A phenomenon similar to the so-called "complement deviation" was observed.

Evidently the action of serum on paramecia is analogous to bacteriolysis and hemolysis. Inactive serum of cold-blooded animals (frog and turtle), however, cannot be complemented by the normal complement either of guinea-pig serum or of frog serum.

Immune rabbit serums contain about 10-15 times as much amboceptor as the normal rabbit serum.

The normal serums of cold-blooded animals have a very much stronger activity than the serum of warm-blooded animals.

The morphologic and physiologic alterations of paramecia observed under the influence of normal and immune serums lead us to conclude that the action of serum on infusoria is typically serologic—a beautiful example of the mechanism and nature of cytolysis. This is a fundamentally different conception from that of Rössle.